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Improving nutritional quality and fungal tolerance in soya bean and grass pea by expressing an oxalate decarboxylase

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Summary

Soya bean (*Glycine max*) and grass pea (*Lathyrus sativus*) seeds are important sources of dietary proteins; however, they also contain antinutritional metabolite oxalic acid (OA). Excess dietary intake of OA leads to nephrolithiasis due to the formation of calcium oxalate crystals in kidneys. Besides, OA is also a known precursor of β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP), a neurotoxin found in grass pea. Here, we report the reduction in OA level in soya bean (up to 73%) and grass pea (up to 75%) seeds by constitutive and/or seed-specific expression of an oxalate-degrading enzyme, oxalate decarboxylase (FvOXDC) of *Flammulina velutipes*. In addition, β-ODAP level of grass pea seeds was also reduced up to 73%. Reduced OA content was interrelated with the associated increase in seeds micronutrients such as calcium, iron and zinc. Moreover, constitutive expression of *FvOXDC* led to improved tolerance to the fungal pathogen *Sclerotinia sclerotiorum* that requires OA during host colonization. Importantly, *FvOXDC*-expressing soya bean and grass pea plants were similar to the wild type with respect to the morphology and photosynthetic rates, and seed protein pool remained unaltered as revealed by the comparative proteomic analysis. Taken together, these results demonstrated improved seed quality and tolerance to the fungal pathogen in two important legume crops, by the expression of an oxalate-degrading enzyme.

Introduction

Oxalic acid (OA), a strong dicarboxylic acid, is naturally abundant in fruits, vegetables and seeds (Holmes and Kennedy, 2000; Holmes *et al.*, 1998). At physiological pH, OA forms soluble salts with sodium and potassium; however, OA chelates calcium to form insoluble calcium oxalate, which renders OA clinically important. Because humans cannot degrade ingested OA due to the lack of any biological and/or enzymatic system, the consumption of high OA-containing vegetable diet is considered detrimental to the human health. This leads to hyperoxaluria and destruction of renal tissues because of the precipitation of calcium oxalate in the kidneys, a medical condition known as nephrolithiasis (Robertson and Peacock, 1980; Sidhu *et al.*, 1999).

Legumes constitute the main source of proteins to the majority of human diets. Legume proteins are rich in essential amino acid lysine, which is lacked in cereals. Besides, legumes are the source of a wide array of essential nutrients, low glycaemic carbohydrates, dietary fibre, vitamins and minerals, and cultivation of legume improves soil health of the agricultural and agroforestry systems. Soya bean (*Glycine max*) and grass pea (*Lathyrus sativus*) are legumes with abundant economical and nutritional importance. Soya bean seeds contain around 38%–40% proteins, whereas beans and/or peas contain nearly 20% proteins (Gwtek *et al.*, 2014). Moreover, soya bean is responsible for providing more than half of globally consumed edible oil (SoyStats, 2013, available at www.soystats.com). Soya bean has become indispensible because it provides a wide range of potential advantages such as protein-based biodegradable materials, anticancerous constituents and potential to lower cholesterol (Ko *et al.*, 2013). Such varied uses of soya bean made it a more desired crop, and its global demand is increasing day by day (Deshmukh *et al.*, 2014). Grass pea (*Lathyrus sativus*) is also an agronomically important crop due to its high protein content, good grain yield, resistance to insects as well as pests. Moreover, it has a potential to tolerate extensive drought and water-logging stresses (Yan *et al.*, 2006; Zambre *et al.*, 2002). This crop is cultivated and consumed in various parts of the world not only for animal feed and fodder but also for human consumption, because it is an inexpensive source of dietary lysine-rich protein for people of low-income food-deficit countries (Barik *et al.*, 2005; Smartt, 1990). However, OA found in soya bean and grass pea seeds is an antinutritional factor (Chai *et al.*, 2004; Yan *et al.*, 2006). Oxalate content of soya bean varies from 0.67 to 3.5 g/100 g of seeds dry weight (Massey *et al.*, 2001). Moreover, OA also serves as a precursor for the biosynthesis of a grass pea neurotoxin, β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) (Xiong *et al.*, 2015). Some of the grass pea germplasms also display a significant variability in

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β-ODAP content, and these grass peas contain 0.5%–2.5% β-ODAP in the seeds (Xiong *et al.*, 2015). Prolonged consumption of *L. sativus* causes neurolathyrism, which is characterized by spasticity of leg muscles, lower limb paralysis, convulsions and death (Yan *et al.*, 2006).

Besides, OA is considered as a pathogenesis factor for the devastating phytopathogenic fungus *Sclerotinia scleriotorum* which synthesizes and secretes OA during host colonization (Kabbage *et al.*, 2015; Malenčić *et al.*, 2010; Uloth *et al.*, 2015). *Sclerotinia sclerotiorum*, a necrotrophic fungal pathogen, instigates white mould of more than 400 plant species, including important crops such as cotton, tomato, sunflower, grass pea and soya bean (Heller and Witt-Geiges, 2013). The pathogenicity of *S. sclerotiorum* is concomitant with OA secretion by fungus, and OA is thought to play a crucial role in infection. The relationship between *Sclerotinia* pathogenicity and OA secretion might heighten *Sclerotinia* virulence mostly by three modes of action (Dutton and Evans, 1996). First, fungus secretes several cell wall-degrading enzymes such as polygalacturonase to invade plant tissues. These enzymes work at low pH, and OA might play an important role in providing the low pH environment for these enzymes. Second, the presence of OA brings down pH of the infection site due to its acidic nature, which further leads to weakening of the plant and thereby facilitates the invasion to plant tissue. Thirdly, because Ca^{2+} is an essential component of plant defence system, OA could bind with Ca^{2+} and form calcium oxalate which may compromise the function of Ca^{2+}-dependent defence. Chelation of cell wall Ca^{2+} by OA might not only compromise the function of Ca^{2+}-dependent defence responses but also weaken the plant cell wall (Bateman and Beer, 1965; Cessna *et al.*, 2000; Dutton and Evans, 1996). This further provides a platform for fungal degradative enzymes to assist the fungal infection (Lumsden, 1979). Moreover, the accumulation of high level of OA during fungal infection results in wilting of the plants and finally cell death (Kim *et al.*, 2008).

Oxalic acid can be biodegraded via two pathways, that is oxidation and decarboxylation. Oxalate oxidase (EC 1.2.3.4), belongs to the germin family of proteins, expressed predominantly in higher plants, catalyses the oxygen-dependent oxidation of oxalate to carbon dioxide in a reaction that is coupled with the formation of hydrogen peroxide. Oxalate oxidase activity is found in a wide variety of plants, including barley seedling, roots and leaves, *Amaranthus* leaves, beet stems and leaves, sorghum leaves and maize, oats, rice and rye (Hurkman *et al.*, 1991; Lathika *et al.*, 1995). *Flammulina velutipes* oxalate decarboxylase (FvOXDC) is thought to be the best characterized oxalate decarboxylase from the wood-rotting fungus reported till date (Chakraborty *et al.*, 2002). FvOXDC is specific to oxalate, and it catabolizes to formic acid (nontoxic organic acid) and CO_2 in a single step without the requirement of a cofactor. This enzyme is active at low pH which would be helpful as most of the oxalate is localized in plant cell vacuoles, where pH is low. FvOXDC is a hexamer of six identical subunits of 410 kDa molecular mass, and the hexamer is a dimer of trimers (Chakraborty *et al.*, 2002). The Mn^{2+} and OA binding to the active site of FvOXDC plays a key role in enzyme catalysis (Chakraborty *et al.*, 2002).

In this present study, we have developed transgenic soya bean and grass pea lines by constitutive and/or seed-specific expression of *FvOXDC* with the aim to reduce OA in soya bean and both OA and β-ODAP in grass pea. The reduction of OA in soya bean and grass pea not only improved their seed nutritional quality but also conferred tolerance to *S. sclerotiorum.*

Results

Expression and stable integration of *FvOXDC* in transgenic soya bean and grass pea

To reduce the levels of antinutritional OA in soya bean and OA and β-ODAP in grass pea, we have generated transgenic lines for both soya bean and grass pea for CaMV35S-driven constitutive expression of *FvOXDC*. Moreover, seed-specific expression of *FvOXDC* was also achieved in soya bean using the seed-specific promoter of soya bean late-embryogenesis-abundant protein (LEA protein; GmPM9). The pSOVA expression plasmid contained *FvOXDC* under the control of the CaMV35S promoter (Figure 1a). In pSPM9 plasmid, *FvOXDC* was under the control of seed-specific promoter (Figure 1h). These two *FvOXDC* constructs (pSOVA and pSPM9) were used to transform soya bean cultivar MACS-57 via *Agrobacterium tumefaciens*-mediated transformation by following a robust and reproducible transformation protocol involving soya bean cotyledonary nods as explants (Figure S1). However, for grass pea, embryos were used as explants (Figure S2) to transform cultivar LP-24. *FvOXDC* gene amplification, following polymerase chain reaction (PCR) using *FvOXDC*-specific primers and genomic DNA as template, was carried out for the kanamycin resistance and single-copy transgene-integrated T3 (soya bean SOVA lines and PSPM9 lines) and T4 (grass pea SOVA lines) generation transgenic lines (Figure 1b,c,i). PCR-amplified DNA fragment of 750 bp was detected in all the kanamycin-resistant transgenic lines, confirming genomic integration of the *FvOXDC*.

*Fv*OXDC protein expression and oxalate decarboxylase activity in transgenic plants

To confirm the expression of *Fv*OXDC in soya bean and grass pea, immunoblot assay was performed using polyclonal anti-*Fv*OXDC antibody. Immunoblot assay using total soluble protein revealed an immunoreactive band of 55 kDa, corresponding to the predicted size of the *Fv*OXDC (Figure 1d,e,j). However, protein band corresponding to the *Fv*OXDC was not detected in the protein extracts of nontransformed (wild-type) plants, confirming specificity of the immunoreactive band. This result confirmed the expression of *Fv*OXDC protein in soya bean and grass pea transgenic lines. Further, to determine the functional expression of *FvOXDC* in soya bean and grass pea, total protein extracts were examined for the OXDC activity. The OXDC activity was examined by measuring the liberation of $^{14}CO_2$ from ^{14}C oxalic acid (Amersham, Little Chalfont, UK). *Fv*OXDC was found to be functionally active with five to sevenfold increase in CO_2 production in transgenic soya bean in pSOVA and pSPM9 *Fv*OXDC-expressing lines, respectively (Figure 1f,g) and two to threefold increase in CO_2 production in transgenic grass pea pSOVA lines (Figure 1k). Moreover, leaves of transgenic and wild type were tested for the OA sensitivity (Foster *et al.*, 2012; Kesarwani *et al.*, 2000). Leaves of *Fv*OXDC-expressing transgenic plants were less chlorotic compared to wild type (Figure 1l). These results confirmed *in planta* role of *FvOXDC* in OA degradation.

Expression of *FvOXDC* decreases OA level in soya bean and OA and β-ODAP level in grass pea

FvOXDC-expressing transgenic soya bean and grass pea lines were assayed for seed OA and β-ODAP content and compared with the nontransformed wild-type plants. The analysis revealed

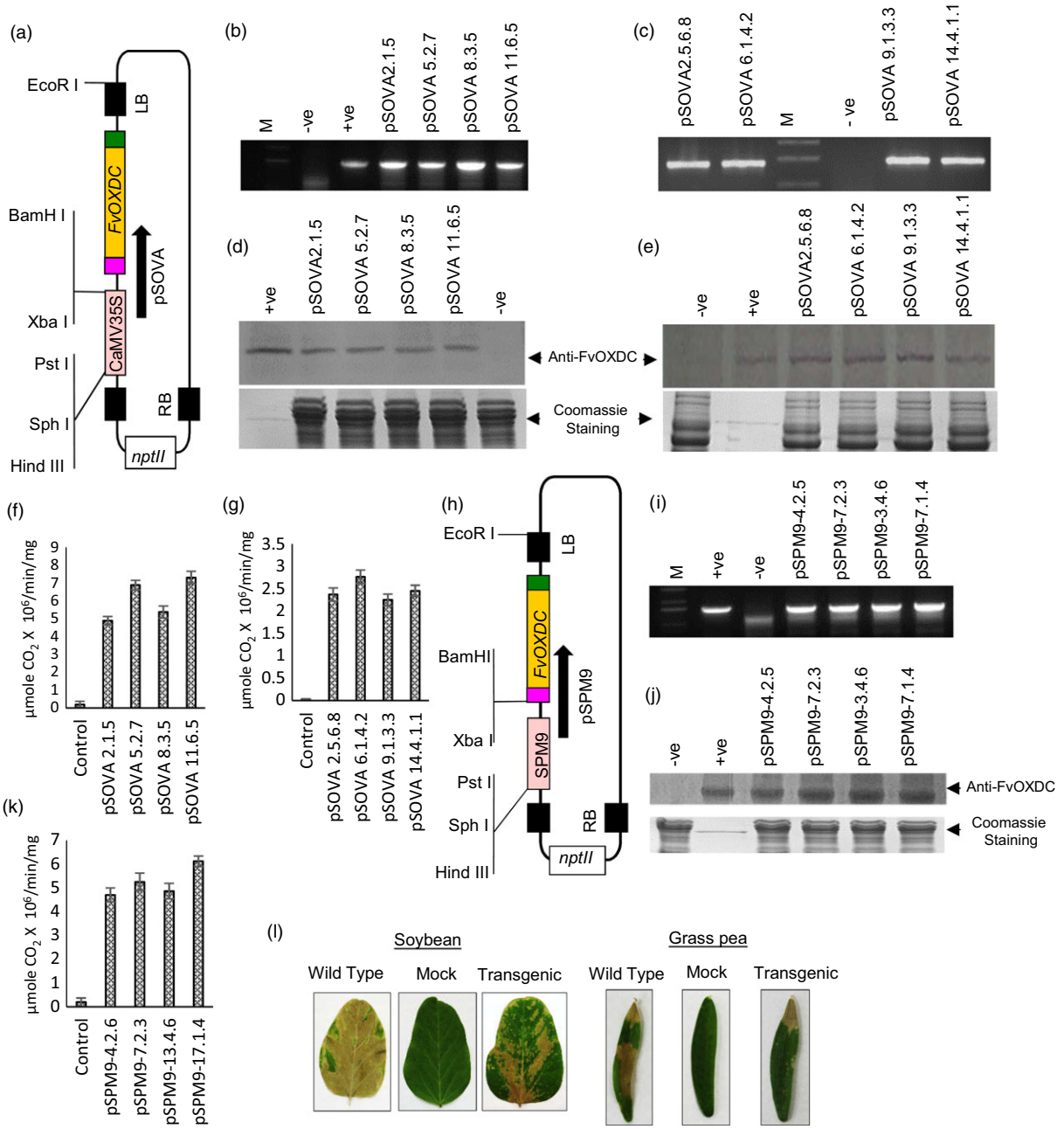

Figure 1 Molecular analyses of soya bean and grass pea transgenic lines expressing *FvOXDC*. (a) Schematic representation of the expression plasmid pSOVA, containing *FvOXDC*-coding sequence under the control of CaMV35S promoter. RB, right border; LB, left border; nptII, neomycin phosphotransferase. (b, c) PCR analysis of transgenic pSOVA soya bean (b) and grass pea (c) lines, for the confirmation of the *FvOXDC* genomic integration. (d, e) Immunoblot analysis to confirm *FvOXDC* expression in transgenic pSOVA lines of soya bean (d) and grass pea (e). (f, g) Oxalate decarboxylase activity shown as micromoles of CO_2 liberated per minute per milligram of protein at 37 °C. Oxalate decarboxylase activities for the pSOVA lines of soya bean (f) and grass pea (g) are shown. Data represent the values obtained from the means (±SE) of ten leaves per plant for each independent transgenic event and the wild type in triplicate. (h) Schematic representation of the expression plasmid pSPM9, containing *FvOXDC*-coding sequence under the control of GmPM9 promoter for seed-specific expression. (i) PCR analysis of transgenic pSPM9 soya bean lines for the confirmation of the *FvOXDC* genomic integration. (j) Immunoblot analysis confirmed *FvOXDC* expression in transgenic pSPM9 lines of soya bean. (k) Oxalate decarboxylase activity of soya bean plants expressing oxalate decarboxylase in a seed-specific manner, shown as micromoles of CO_2 liberated per minute per milligram of protein at 37 °C. Data represent the values obtained from the means (±SE) of ten seeds per plant for each independent transgenic event and the wild type in triplicate. (l) Oxalic acid (OA) sensitivity of the wild type and transgenic plants constitutively expressing *FvOXDC* was compared. Excised leaves were immediately dipped in the OA solution, and a negative control was kept with wild-type leaf in water (mock).

up to 67% reduction in OA level in pSOVA lines (Figure 2a) and up to 73% reduction in pSPM9 lines of soya bean (Figure 3a), whereas OA and β-ODAP contents of grass pea were reduced up to 75% and 73%, respectively (Figure 4a,b). As *Fv*OXDC-mediated decarboxylation of OA results in the generation of formic acid, the levels of formic acid in the seeds of *FvOXDC*-

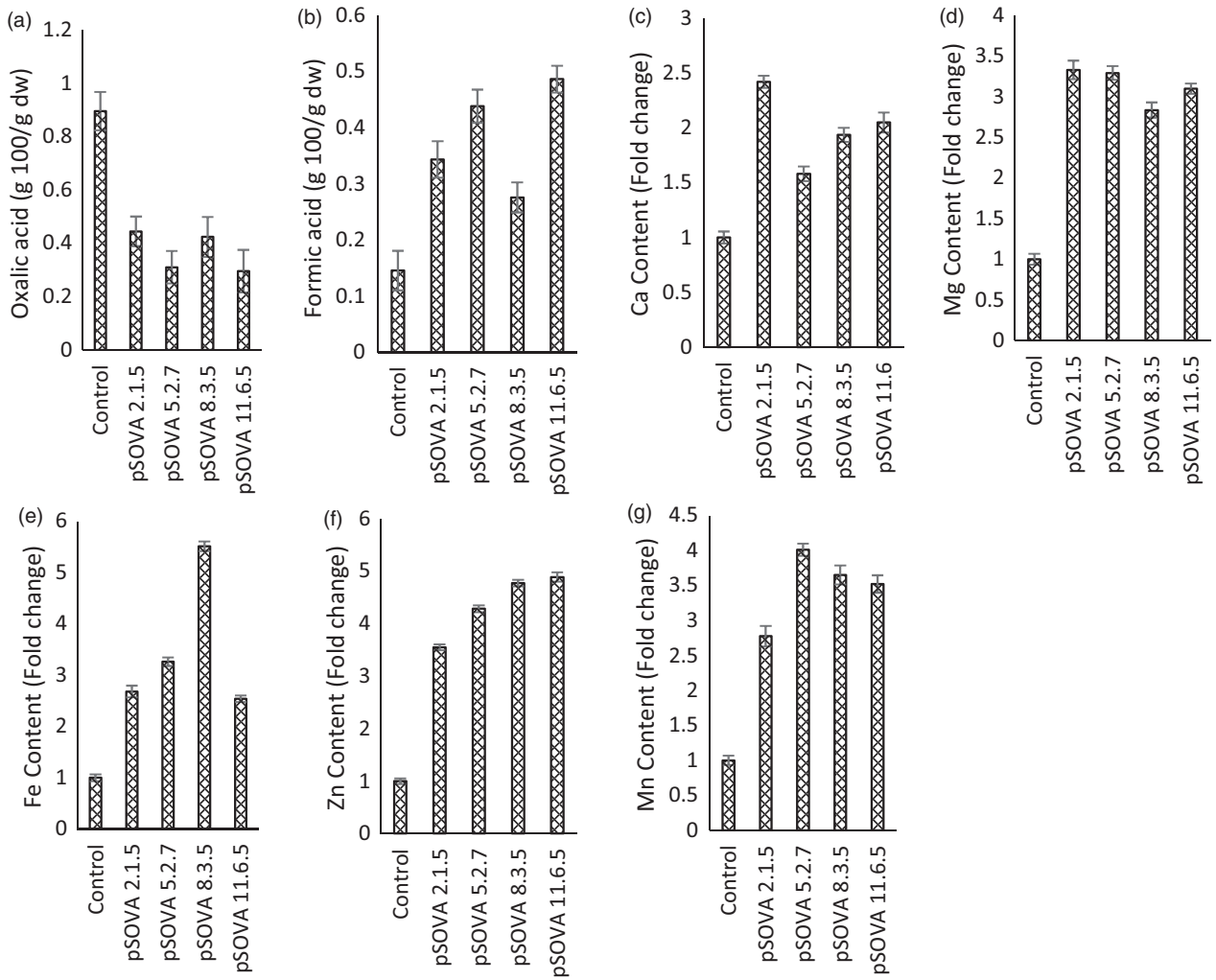

Figure 2 Biochemical analyses of transgenic soya beans constitutively expressing *FvOXDC*. (a, b) Quantitation of OA and formic acid in soya bean seeds expressing *FvOXDC* under the control of CaMV35S promoter. Levels of calcium (c), magnesium (d), iron (e), zinc (f) and manganese (g) were determined using energy-dispersive X-ray fluorescence spectrophotometry in soya bean transgenic lines expressing *FvOXDC* in a constitutive manner. Data represent the values obtained from the means (±SE) of ten seeds per plant for each independent transgenic event and the wild type in triplicate.

expressing transgenic lines were also determined. A concomitant increase (70%) in formic acid was observed with the expression of *FvOXDC* in soya bean transgenic lines (Figures 2b and 3b). Thus, *FvOXDC* expression was correlated with the reduced level of OA in soya bean and grass pea and reduced β-ODAP level in grass pea.

Reduction in OA content resulted in increased levels of some micronutrients

Micronutrients play a crucial role in human and animal health, and thus, it is desired to generate transgenic plants with higher levels of these components (Gupta *et al.*, 2015; Hefferon, 2015). Because OA is a strong chelator of divalent cations, especially calcium, therefore, we were interested to determine the micronutrient contents in *FvOXDC*-expressing transgenic soya bean and grass pea seeds. Calcium and other micronutrients such as magnesium, zinc, iron, sodium and potassium were analysed by energy-dispersive X-ray fluorescence analysis (EDXRF). A marked increase in the levels of calcium, magnesium, zinc, iron and manganese in the seeds was recorded. Calcium level increased more than two folds in low-oxalate transgenic pSOVA and pSPM9 lines of soya bean (Figures 2c and 3c). In the seeds of both pSOVA and pSPM9 line, a significant increase in the levels of other nutrients such as Mg, Fe, Zn and Mn was also noticed in comparison with the control seeds (Figures 2d–g and 3d–g). The content of calcium, magnesium, zinc, iron and manganese in grass pea seeds was also significantly increased (Figure 4c–g). These data suggested an increased concentration of micronutrients in the seeds of low-oxalate transgenic soya bean and grass pea.

Reduced OA content led to enhanced tolerance to *S. sclerotiorum*

Sclerotinia sclerotiorum pathogenicity is associated with OA (Dutton and Evans, 1996). Therefore, we tested the response of transgenic soya bean and grass pea, constitutively expressing *FvOXDC*, to the *S. sclerotiorum* infection. Transgenic soya bean and grass pea and wild-type plants were infected with *S. sclerotiorum* as described in Experimental procedures using mycelia suspension, and the progression of disease symptoms was monitored for different time intervals. Inoculated leaves of transgenic and wild types were considered as infected with

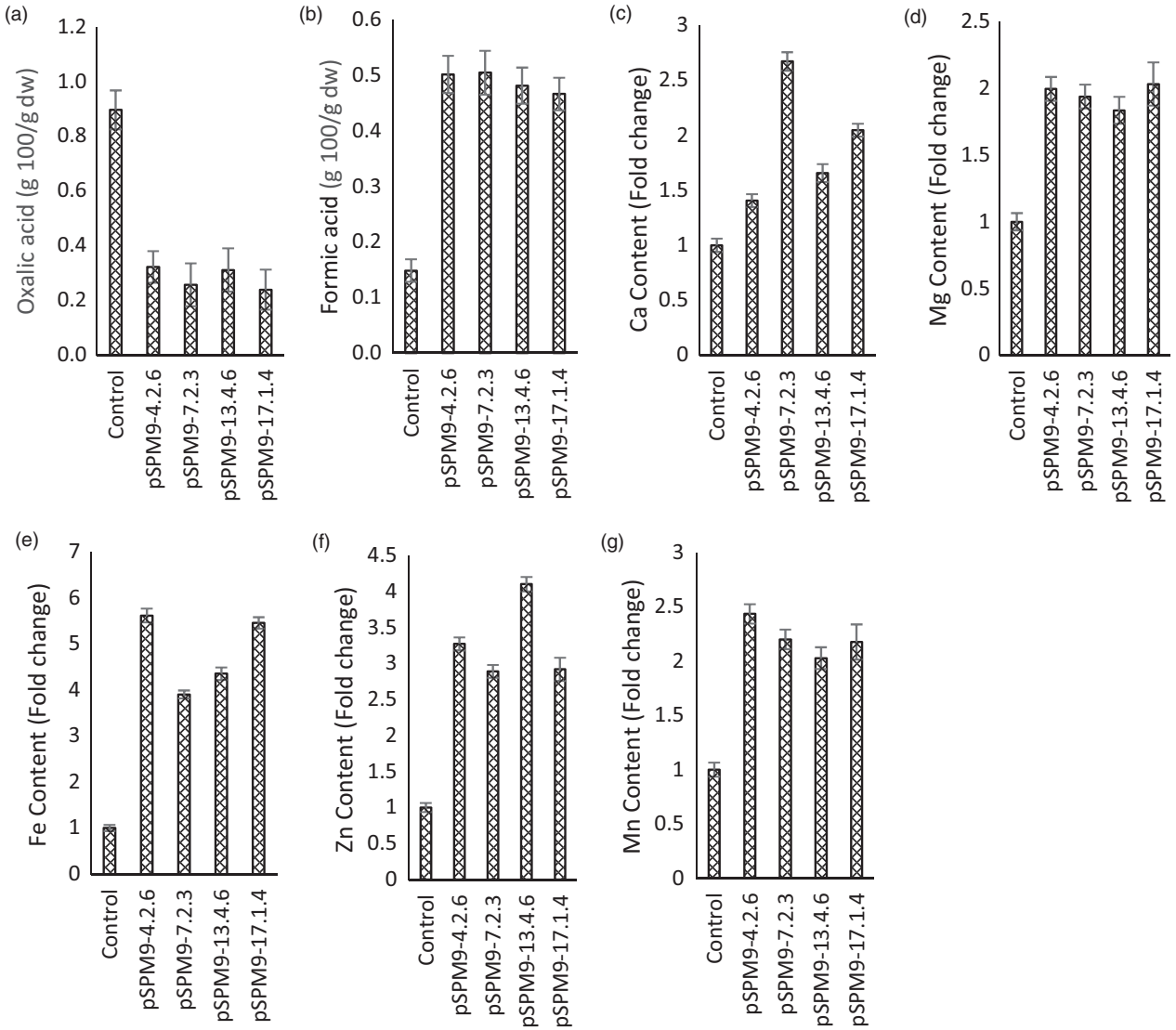

Figure 3 Biochemical analyses of transgenic soya beans expressing *FvOXDC* in a seed-specific manner. (a, b) Quantitation of OA and formic acid in soya bean seeds expressing *FvOXDC* under the control of pSPM9 seed-specific promoter. Levels of calcium (c), magnesium (d), iron (e), zinc (f) and manganese (g) were determined using energy-dispersive X-ray fluorescence spectrophotometry. Data represent the values obtained from the means (±SE) of ten seeds per plant for each independent transgenic event and the wild type in triplicate.

S. sclerotiorum when the symptoms of tissue browning and lesion formation appeared. Compared to the nontransgenic wild type, *FvOXDC*-expressing transgenic leaves displayed a substantial delay in the lesion progression (Figure 5a,b). Lesion intensity was also low in transgenic pSOVA lines of both soya bean and grass pea as compared to their corresponding wild-type plants (Figure 5c,d).

Quantitative proteomic analysis revealed unaltered protein profile in the seeds of transgenic plants

Seeds of transgenic soya bean and grass pea were examined for their protein profile using isobaric tags for relative and absolute quantitation (iTRAQ) analysis and compared to the wild type as described in Experimental procedures. To understand protein profile of the transgenic and wild-type seeds, 4 plex iTRAQ labels were used. Proteins of the wild type were labelled with 114 and 115, whereas proteins of the transgenic soya bean were labelled with 116 and 117. In case of grass pea, proteins isolated from wild type were labelled with 114 and 115, whereas proteins isolated from transgenic grass pea were labelled with 116 and 117. iTRAQ analysis revealed unaltered protein profiles in the seeds of transgenic and wild-type soya bean and grass pea (Figure S3a,b and Tables S1 and S2).

Transgenic soya bean and grass pea resembled wild-type plants with respect to growth and developmental phenotype and physiology

Transgenic soya bean and grass pea were compared with wild-type plants, either in the primary transgenic populations or in the subsequent generations, and there were no observable differences in phenotype, development or fertility in any of five independent transgenic line of each soya bean (pSOVA, pSPM9) and grass pea (pSOVA) transgenic lines. Transgenic lines were also checked for their field performance by transplanting them in the experimental field alongside wild-type plants and randomly replicated for further generations. Transgenic plants were normal

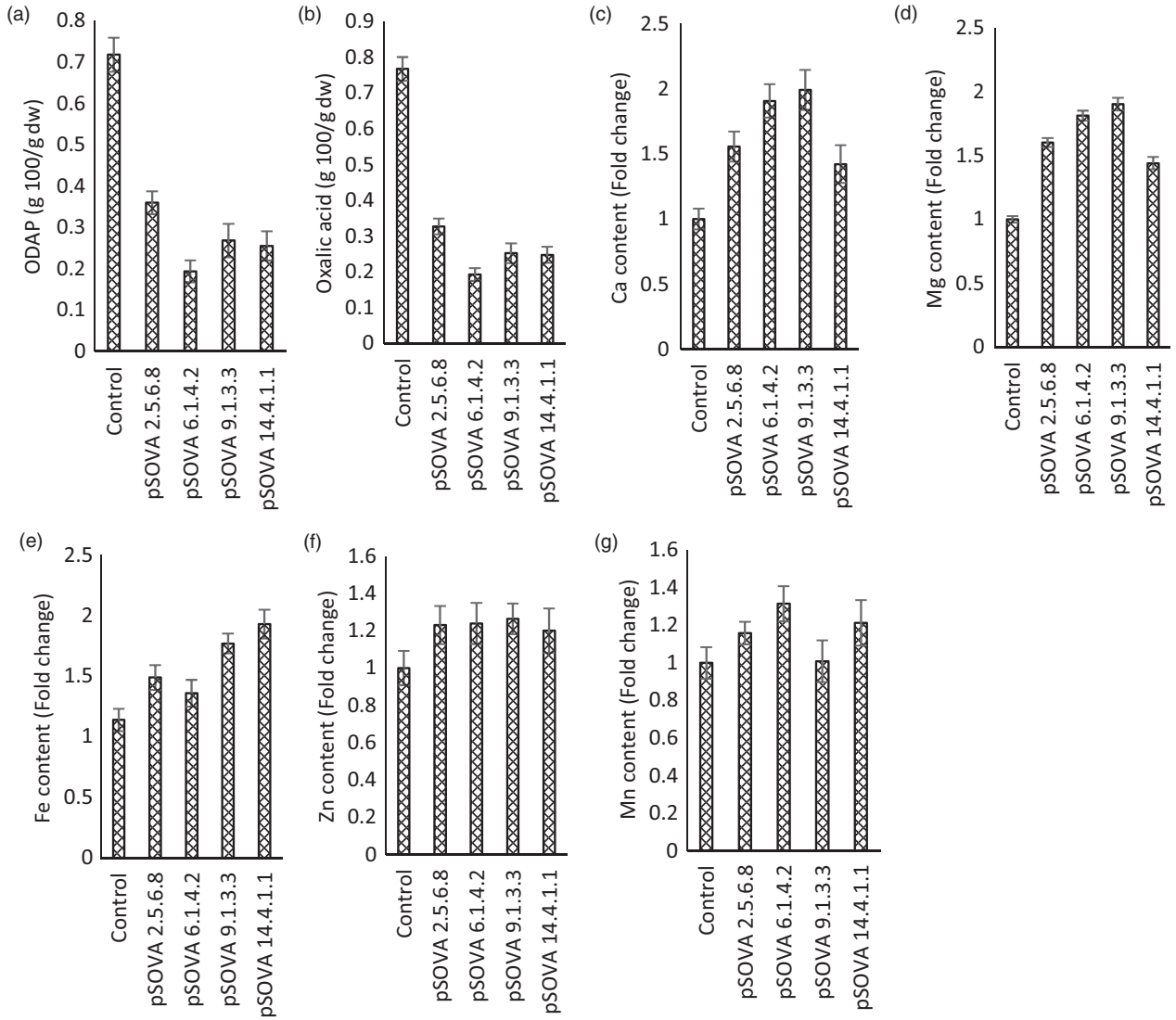

Figure 4 Biochemical analyses of transgenic grass pea lines constitutively expressing *FvOXDC*. (a, b) Quantitation of ODAP and OA in grass pea expressing *FvOXDC* in a constitutive manner. Levels of calcium (c), magnesium (d), iron (e), zinc (f) and manganese (g) were determined using energy-dispersive X-ray fluorescence spectrophotometry in grass pea transgenic lines expressing *FvOXDC* in a constitutive manner. Data represent the values obtained from the means (±SE) of ten seeds per plant for each independent transgenic event and the wild type in triplicate.

in vegetative growth, and their morphology was normal as compared to the wild-type plants. Transgenic plants were similar in flowering time, vigour or total yield as observed for wild type. Moreover, expression of *FvOXDC* did not affect the net photosynthetic rates or other physiological parameters, including stomatal conductance and transpiration rate.

Discussion

With long exposure of plant breeding techniques and genetically modification through transgenic technology, there has been a substantial success in augmenting the nutritional value of food crops. Efforts have been made to improve the quality and quantity of macronutrients such as protein/amino acids, oils/fats and carbohydrates as well as to increase the bioavailability of micronutrients, vitamins and antioxidants through transgenics and breeding. In soya bean, most of the work was carried out to improve seed protein quality (Cunha *et al.*, 2011; Nishizawa *et al.*, 2010; Qi *et al.*, 2011), oil content (Kajikawa *et al.*, 2008; Li *et al.*, 2010), biotic resistance (Cunha *et al.*, 2010; Dang and Wei, 2007; McLean *et al.*, 2007; Tougou *et al.*, 2007) and abiotic resistance (DeRonde *et al.*, 2004; Valente *et al.*, 2009). However, on the other hand, there have been limited efforts in the management of antinutrients and/or toxins, which are the by-products of plant metabolism and considered as health hazards to humans and animals. OA is one of the known antinutritional factors, which has negative health concerns, when we consume crops containing high amount of OA, such as tomato, spinach, soya bean and grass pea.

Legumes are very challenging to regenerate and transform because somatic embryogenesis or organogenesis is found to be difficult (Iantcheva *et al.*, 2013). Here, in the present study, we have developed an efficient transformation method for both soya bean and grass pea using cotyledonary explants and embryo explants, respectively (Figures 1, S1 and S2). Further, to improve the nutritional quality of soya bean and grass pea by down-

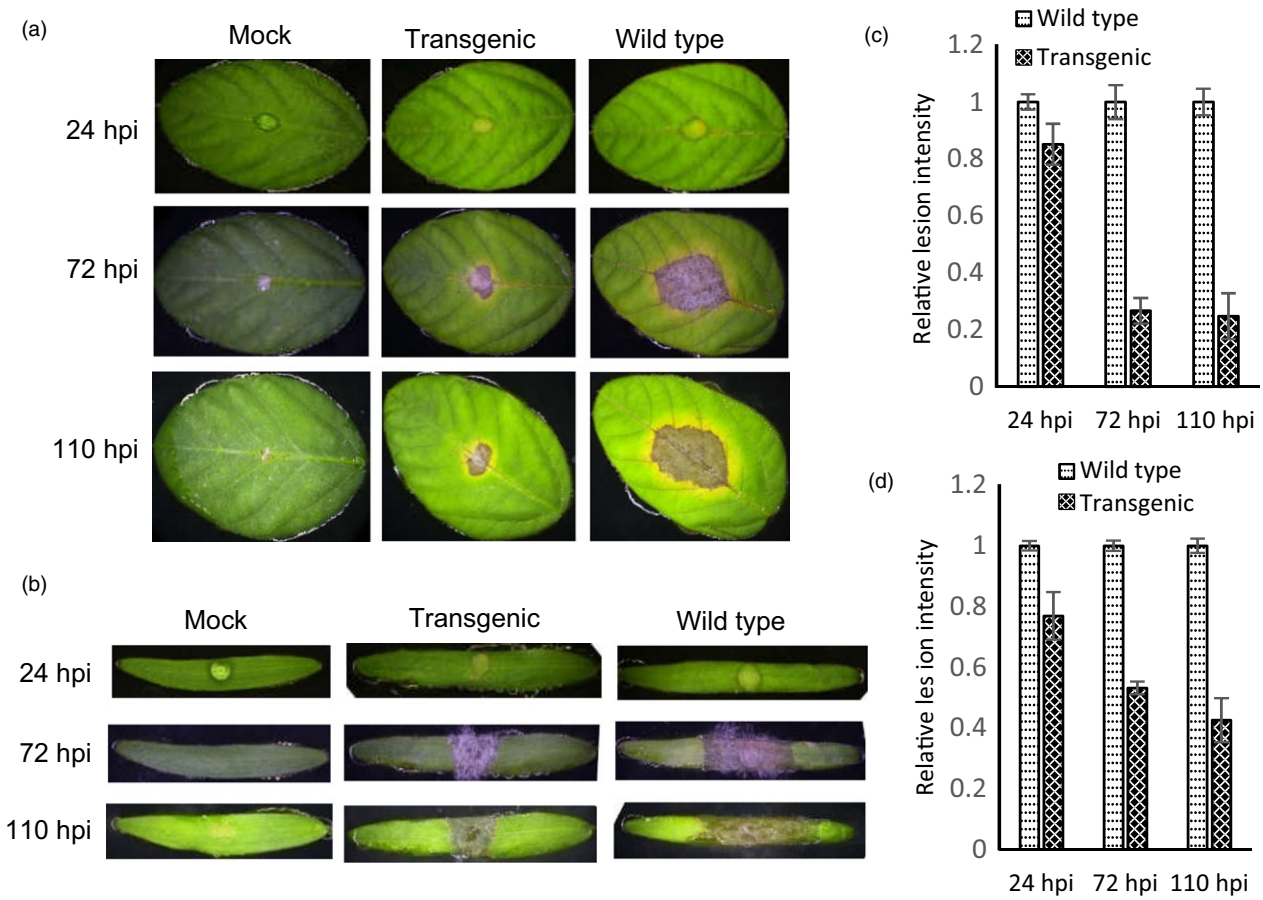

Figure 5 Transgenic soya bean and grass pea plants showed an enhanced tolerance to *S. sclerotiorum* infection. (a, b) Leaves of transgenic and wild-type plants of soya bean (a) and grass pea (b) were inoculated with equal amount of mycelial suspension as described under Experimental procedures, and progression of the disease symptom was observed for 110 h postinoculation (hpi). (c, d) Relative lesion intensity was presented for the transgenic and wild-type plants of soya bean (c) and grass pea (d).

regulating OA, we have developed transgenic plants expressing a well-characterized *FvOXDC* (Azam *et al.*, 2001, 2002; Chakraborty *et al.*, 2002, 2013; Kesarwani *et al.*, 2000). Our results showed that there was a significant decrease in OA content in both the lines of pSOVA and pSPM9 (Figures 2a and 3a). Transgenic soya bean plants expressing *FvOXDC* in a seed-specific manner were superior in OA management than the constitutive lines. OA and β-ODAP contents of grass pea were also assayed biochemically, and their contents were found to be reduced (Figure 4a,b). Expression of *FvOXDC* in soya bean and grass pea not only led to a significant reduction in OA levels but also associated with a substantial increase in calcium, conceivably by down-regulating the content of OA that chelates calcium (Figures 2c, 3c and 4c). We have also found an increase in other elements such as iron, manganese, magnesium and zinc in transgenic lines of soya bean and grass pea (Figures 2d–g, 3d–g and 4d-g). Previous reports suggested that the expression of Ca^{2+}/H^{+} exchangers can alter the sum of all the mineral nutrients (Cheng *et al.*, 2005; Mei *et al.*, 2007). However, in *FvOXDC*-expressing transgenic lines, the increase may be related to the high turnover of OA, which ultimately leads to an increase in these elements.

Sclerotinia sclerotiorum is one of the most devastating, broad-based, necrotrophic, soilborne plant pathogens, which causes stem rot or white mould, with a host range of more than 400 species of plants, comprising crops, ornamental plants, trees and several weeds (Malenčić *et al.*, 2010). Studies with OA-deficient mutants have shown that this molecule is a key suppressor of plant defences, thus allowing the fungus to evade recognition (Williams *et al.*, 2011). Therefore, the ability of *S. sclerotiorum* to infect *FvOXDC*-expressing low OA-containing transgenic soya bean and grass pea was tested. Infection assay performed with detached leaf revealed a noteworthy delay in lesion development and more tolerance to *S. sclerotiorum* infection by the *FvOXDC*-expressing transgenic lines compared to the wild type (Figure 5). These results also demonstrated that the generation of fungal-tolerant soya bean and grass pea lines is also achievable by expressing *FvOXDC*. Previously, various transgenic plants, which can metabolize and/or degrade OA in an enzymatic manner, have been generated and shown to have enhanced tolerance to *S. sclerotiorum* (Cunha *et al.*, 2010; Donaldson *et al.*, 2001; Hu *et al.*, 2003; Kesarwani *et al.*, 2000; Walz *et al.*, 2008). OA treatment suggested that FvOXDC is active *in vivo* as transgenic leaves of both soya bean and grass pea could resist the formation of brown lesion and yellowing caused by OA (Figure 1l).

Previous reports suggested that OA suppresses host defence by manipulating redox status (Cessna *et al.*, 2000; Lehner *et al.*, 2008; Williams *et al.*, 2011). At the initial stage of disease establishment, OA induces a reducing environment in the host cells that suppresses host defence-related oxidative burst and

callose deposition. However, at the later stage, OA promotes the generation of ROS in host tissue leading to programmed cell death which directly benefits the necrotrophic pathogens (Williams *et al.*, 2011). On the contrary, nonpathogenic OA-deficient mutant is unable to alter host redox status (Cessna *et al.*, 2000; Williams *et al.*, 2011). Moreover, the effect of OA on host defence response might be mediated through phytohormone signalling pathways such as jasmonic acid (JA), ethylene (ET), abscisic acid (ABA) and salicylic acid (SA). It was suggested that OA induces JA, ET and ABA signalling pathways; however, it suppresses SA pathway in *Brassica napus* (Liang *et al.*, 2009). Similarly, overexpression of OA-responsive WRKY family transcription factors revealed that JA/ET pathway is mainly active to defend *Arabidopsis* against *S. sclerotiorum* infection and OA stress (Chen *et al.*, 2013). These observations are in agreement with an important role of the host JA/ET signalling pathway in response to necrotrophic fungal pathogens (Liang *et al.*, 2008; Van Kan, 2006; Yang *et al.*, 2007; Zhao *et al.*, 2007). Although we have tested *S. sclerotiorum* tolerance of the *FvOXDC*-expressing transgenic lines of soya bean and grass pea (Figure 5), these transgenic lines might also exhibit an altered tolerance to other necrotrophic fungal pathogens such as *Alternaria alternaria, Botrytis cinerea.* Interestingly, manipulating host OA level through exogenous treatments has been shown to affect the infectivity of *A. alternaria* and *B. cinerea* (Schoonbeek *et al.*, 2007; Tian *et al.*, 2006).

To investigate the performance of the *FvOXDC*-expressing soya bean and grass pea, we have analysed the agronomic attributes of transgenic plants and compared with the wild type. Visible morphological changes such as plant height, leaf pattern and morphology, flowering pattern and timing, pod and seed setting as well as number of pods per plant and number of seeds per pod could not be recognized between wild type and *FvOXDC*-expressing lines. Although the seeds of transgenic lines accumulated higher level of minerals than the wild type, it did not affect the plant growth and development (Figures 2–4). In a previous study, fruit-specific expression of *FvOXDC* in transgenic tomato lines was found similar in growth and development with their wild-type counterpart irrespective to a significant increase in mineral contents of fruits (Chakraborty *et al.*, 2013). Other studies also supported that increase in calcium content in transgenic plants did not affect the plant growth in tomato, potato and lettuce (Chung *et al.*, 2010; Kim *et al.*, 2006; Morris *et al.*, 2008; Park *et al.*, 2004, 2009). Moreover, transgenic indica rice developed by endosperm-specific expression of soya bean *ferritin* found to contain higher iron and zinc levels and showed normal phenotype, morphology and fertility (Vasconcelos *et al.*, 2003). Altogether, these results suggested that the accumulation of mineral in seeds of the transgenic lines might not have an adverse effect on plant growth and development.

It was also important to determine seed proteins qualitatively and quantitatively to exclude any unintentional changes that may occur in transgenic seeds as a result of this genetic modification. Proteomic technology is very worthwhile to investigate protein variation due to innumerable changes during the transgenic modification of a crop (Natarajan *et al.*, 2013). We have also evaluated the protein profiles of soya bean and grass pea seeds, using proteomics' iTRAQ approach, because seeds or seed-derived products are most consumed for these crops. Most of the proteins identified were checked for their differential expression, and we found no remarkable expression difference in the identified proteins (Figure S3a,b and Tables S1 and S2). To determine how a transgene might affect global gene expression or metabolism of the host plant, evaluation of transgenic plants can be performed at the levels of transcriptome, proteomics and metabolome (Ricroch *et al.*, 2011). Previously, transcriptome profiling of about 24 000 genes of transgenic *Arabidopsis* expressing *nptII/uidA* revealed no noticeable difference with the wild type (El Ouakfaoui and Miki, 2005). Similarly, many researchers performed profiling of transcripts, proteins and metabolites of transgenic plants without any significant differences (Barros *et al.*, 2010; Cheng *et al.*, 2008; Coll *et al.*, 2009; Kogel *et al.*, 2010). In conclusion, we have developed genetically modified soya bean and grass pea with improved nutritional quality and enhanced tolerance to fungal pathogen without any adverse effect on plant growth and development and seed protein quality.

Experimental procedures

Construction of *FvOXDC* expression vectors

Overexpression of *FvOXDC* in soya bean and grass pea was performed using pSOVA (for soya bean and grass pea) and pSPM9 (for soya bean) constructs. To overexpress *FvOXDC* specifically in soya bean seeds, the coding sequence of *FvOXDC* was cloned under the regulation of the promoter of a soya bean gene encoding LEA protein GmPM9 by replacing the CaMV35S promoter of pSOVA (Kesarwani *et al.*, 2000) with the GmPM9 promoter (Lee *et al.*, 2000). pSPM9 was mobilized into *Agrobacterium tumefaciens* strain EHA105 using the helper strain HB101:: pRK2013 via the triparental mating technique (Van Haute *et al.*, 1983).

Transformation and selection of transgenic events of soya bean

Cotyledonary node explants were excised from 7- to 10-day-old seedlings germinated on germination medium [Gamborg's B-5 basal medium (G5893) with 3% sucrose and 1 mg/L benzyl amino purine (BAP), pH adjusted to 5.6–5.8]. After agro-infection, cotyledonary node explants were transferred to co-cultivation medium [Gamborg's B-5 basal medium (G5893) with 3% sucrose, 200 μM acetosyringone, 0.25 mg/L gibberellic acid (GA3), 1 mM dithiothreitol (DTT), 1 mM sodium thiosulphate (STS), pH adjusted to 5.6] and maintained for 5 days. Further, explants were transferred to shoot induction and selection medium [Gamborg's B-5 basal medium (G5893), 3% sucrose] supplemented with 1.5 mg/L BAP, 0.25 mg/L GA3, 250 mg/L cefotaxime and 75 mg/L kanamycin. Three- to 7-cm-long shoot buds were transferred to rooting media consisting of ½ MS basal media with 3% sucrose, 0.5 mg/L IAA, 50 mg/L kanamycin and 250 mg/L cefotaxime, pH 5.8. Plantlets with well-developed roots were taken out from the medium, and the roots were rinsed in water to remove agar and transferred to small pots containing sterilized agro peat and vermiculite (2 : 1).

Transformation and selection of transgenic events of grass pea

Embryo explants were used to transform *FvOXDC* gene to grass pea. Explants were excised from overnight-soaked seeds and then agro-infected and transferred to co-cultivation medium (Gamborg's B-5 basal medium, 3% sucrose, 0.5 mg/L NAA, 0.5 mg/L BAP, 1 mM DTT, 1 mM sodium thiosulphate and 0.2 mM acetosyringone, pH 5.6) and incubated at 23 °C for 5 days.

Further, explants were transferred to shoot induction medium (Gamborg's B-5 basal medium, 3% sucrose with 0.5 mg/L NAA and 1 mg/L BAP, pH 5.6–5.8) and selected on 100 mg/L kanamycin. Subculturing was performed at an interval of 15 days on shoot induction medium till shoots appeared. Small plantlets were excised and transferred to root induction medium (Gamborg's B-5 basal medium with 3% sucrose, 0.1 mg/L IBA, pH 5.6) for rooting. Transformed plants were maintained in the growth room before being transferred to the experimental plot.

Nucleic acid isolation and analysis

To confirm the integration of *FvOXDC* in the putative transgenic plant genome, genomic DNA was extracted from leaf tissue (100 mg) using the DNeasy plant mini kit (Qiagen, Duesseldorf, Germany). The stable integration of the *FvOXDC* gene in the transgenic soya bean and grass pea plants was determined via PCR using the forward and reverse primers (5′-GCGAAGTGGC-CATTCAGCTTCAGC-3′) and (5′-CAGCTCTCTCAAAGCAC-CAGGCTC-3′), respectively.

Immunodetection of FvOXDC

Crude proteins were extracted from soya bean and grass pea transgenic plants and their respective controls. From each sample, 100 μg of total protein was then separated on a 12.5% SDS–polyacrylamide gel electrophoresis. Proteins from SDS–PAGE gel were transferred onto nitrocellulose Hybond membrane by electrotransfer (GE Biosciences, Little Chalfont, UK). The membrane was probed with affinity-purified oxalate decarboxylase polyclonal antibody in combination with alkaline phosphatase-conjugated goat anti-rabbit IgG, using NBT-BCIP reaction.

Decarboxylation assay

The decarboxylation activity of FvOXDC was determined using ^{14}C-oxalic acid (GE Biosciences) as a substrate using a method described previously (Mehta and Datta, 1991). 100 μg of soluble protein from each biological replicate was used for the decarboxylation assay. Samples were analysed in triplicate, and the mean value was calculated. One unit of decarboxylase activity is equivalent to 1 μmol/m ^{14}C-CO_2 produced at 37 °C. The activity data were expressed as the means ± SD of at least three independent experiments.

OA treatment to leaves

The assay was performed according to Kesarwani *et al.* (2000) with slight modifications. Young leaves (three each) were detached from transgenic (soya bean/grass pea) and control (soya bean/grass pea) plants. Both transgenic and control leaves were then immediately dipped in 20 mM OA solution pH 4.0. Negative control was imposed by dipping the leaves in water. All the leaves were incubated in a plant growth chamber at 22–24 °C under 16-h/8-h photoperiod for 24 h.

Extraction and determination of OA and formic acid

Oxalic acid and formic acid were quantified using OA and formic acid detection kit according to the manufacturer's protocol (Boehringer Mannheim/R-Biopharm, Darmstadt, Germany). Briefly, each sample was crushed into fine powder using mortar and pestle and then further suspended in 500 μL of MQ water. Homogenization was performed using a PRO 200 homogenizer for five cycles of 30 s each. Homogenate was then centrifuged at 13 000 ***g*** for 15 min at room temperature, and the supernatant was collected. Oxalate and formate contents were measured using 100 μL of the supernatant in triplicate, and the absorbance was read at 590 and 340 nm, respectively.

Determination of β-ODAP

Estimation of β-ODAP content from the transgenic and control plants was performed according to Rao (1978). To 100 μL of seed extract, 200 μL of 3 N KOH was added and the tubes were placed on boiling water bath for 30 min. After alkali hydrolysis, 700 μL of water was added to the tubes so that the final volume of the reaction is 1 mL followed by the addition of 2 mL *o*-phthalaldehyde (OPT) reagent. Samples were mixed and allowed to stand for 15 min to develop yellow colour. The absorbance was measured at 420 nm.

Determination of calcium and other micronutrients

Total calcium and other micronutrients such as magnesium, zinc, iron and manganese were analysed by energy-dispersive X-ray fluorescence analysis (EDXRF; PANalytical Epsilon 5, Almelo, The Netherlands). EDXRF gives unique spectra for each sample analysed. Seeds were grounded into fine powder and dried; then, 60 mg of dried seed powder was used to analyse the total calcium, magnesium, zinc, iron and manganese. A specific X-ray fluorescence signal emitted by the atoms after the photoelectric ionization was measured, and the radiation intensity of each element signal, which is proportional to the concentration of the element in the sample, is recalculated internally from a stored set of calibration curves and can be shown directly in concentration units.

Morphological characterization and photosynthetic activity

Plants were grown in the field, and the experiment was arranged in a randomized block design with three replicates. Plants were regularly watched for their morphological characters such as plant height, size of leaves, number of pods per plant and number of seeds per pod. Photosynthetic rates were measured with a GFS3000 portable photosynthesis measurement system (Walz, Effeltrich, Germany) after 60 days since they were planted. The experiment was performed at standard atmospheric (360 ppm CO_2), light conditions (750 μmol/m^2/s), and humidity was maintained at 70%. Photosynthetic rate of the leaf was measured on the basis of single-leaf measurement experiment which was conducted on three different leaves in each plant.

Detached leaf assays

To analyse the *S. sclerotiorum* infection in transgenic with respect to wild type, detached leaf assay was performed according to Cunha *et al.* (2010) with minor modifications. *S. sclerotiorum* were grown in potato dextrose broth (PDB) at 22 °C with 150 rpm till the growth of mycelial ball. Then, 2 g of mycelial ball was homogenized into 4 mL of ½ PDB and 10 μL of mycelial suspension was inoculated on detached leaves of transgenic soya bean and grass pea as well as to their wild-type counterparts. Leaves were photographed at three different times (24, 72 and 110 h), and images were used to measure the infected area using the NIS Element AR 3.2 64-bit software of multiobjective stereo zoom (AZ-100) microscope (Nikon Instruments Inc., Melville, NY, USA).

Protein isolation, trypsin digestion, iTRAQ 4-plex labelling and data analysis

Total proteins were isolated from soya bean and grass pea seeds using Plant Total Protein Extraction Kit (Sigma-Aldrich, St Louis,

MO, USA, Catalog number PE0230) according to the manufacturer's instructions. For proteomic analysis, biological duplicates from each of the two conditions (wild type and transgenic line) studied were handled simultaneously for iTRAQ labelling (AB Sciex, Foster City, CA) as per the manufacturer's instruction. Briefly, 70 μg of protein from each sample was used for modified trypsin (Promega, Madison, WI, USA, V511) and incubated for 16 h at 37 °C in a 1 : 10 trypsin-to-protein ratio (Maity *et al.*, 2014). To label trypsin-digested peptides of each sample, different iTRAQ tags such as 114, 115, 116 and 117 were used following the manufacturer's protocol (AB Sciex). The data were assimilated in an information-dependent acquisition (IDA) mode Analyst TF 1.6 software (AB Sciex) using the triple ToF 5600 (AB Sciex). For the identification of proteins, Paragon algorithm in a 'Thorough ID' search mode was employed against the SGD-Saccharomyces cerevisiae reference data set (6750 protein sequences). The search parameters allowed modifications by IAA at cysteine residues, 4 plex peptide iTRAQ labelling of the N-termini of peptides and of the side chains of lysine. We applied 1% global protein-level FDR for protein identification.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Transformation and regeneration of soya bean cotyledonary nodes under kanamycin selection, and recovery of transgenic plant.

Figure S2 Transformation and regeneration of grass pea embryos as explants under kanamycin selection, and recovery of transgenic plant.

Figure S3 Protein profiling of soya bean and grass pea seeds with iTRAQ technique.

Table S1 List of soya bean proteins identified and quantified through iTRAQ.

Table S2 List of grass pea proteins identified and quantified through iTRAQ.